UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

005224

JUN 20 1986

OFFICE OF
PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: Ethalfluralin
N-ethyl-N-(2-methyl-2-propenyl)-2,6-dinitro-
4-(trifluoromethyl)benzenamine.

TOX CHEM 453B

FROM: Ray Landolt
Review Section #1
Toxicology Branch/HED (TS-769) 6/25/86

TO: Richard Mountfort, PM #23
Registration Division (TS-767)

THRU: Robert B. Jaeger, Section Head
Review Section #1
Toxicology Branch/HED (TS-769) 6/27/86 6/39/86

Registrant: Elanco Products Company letters of September 12, December 2, 1985, and May 8, 1986

Registration No.: 1471-122

Action Requested:

Review the following toxicity studies identified as conditional requirements in the Registration Notice dated November 28, 1983.

I. Genetic Toxicity Studies

A. Chromosome Abberations in Chinese hamster ovary (CHO) cells in vitro.

B. Forward mutation in Schizosaccharomyces pombe P-1 Cells.

C. A summary of genetic toxicology studies with ethalfluralin.

II. Teratology in the rat.

III. One-year dog oral toxicity study.

Data Evaluation

I. Genetic toxicity studies

A. The chromosome abberation in Chinese hamster ovary (CHO) cells in vitro study is acceptable.

B. Gene mutation in Schizosaccharomyces pombe P-1 cells (LSR-RTC No. 095005-M-03085) is unacceptable in the present form and may be upgraded to acceptable on resolution of the reporting deficiencies.

C. From the summary of genetic toxicity studies submitted the dominant lethal test in rats (R-159) and the DNA repair in bone marrow (No. 83021 4SCE1169) study are deficient. Until the reporting deficiencies cited for these studies are clarified and resolved the potential genotoxicity of ethalfluralin in mammalian systems cannot be determined.

II. The teratology study in the rat has satisfied the toxicity data gap for a second teratogenic study cited in the review of R. Landolt, October 30, 1985.

III. The following deficiencies were observed with the 1-year dog oral toxicity study.

A. Spleen organ weights were not recorded.

B. Bone marrow evaluation for animal number 183623 was not reported.

Recommendation:

From the effects reported on the hematological parameters investigated in the dog study, an anemic trend is apparent from the oral administration of daily doses of ethalfluralin to dogs for 1 year. These hematogical changes at the 20 and 80 mg/kg levels are associated with alterations in erythrocyte morphology and increased erythoid series of the bone marrow.

With reference to the literature for the effects observed on erythropoiesis in comparison with structurally related dinitro chemicals, a rationale for the omission of spleen organ weights should be provided in order to determine if the resulting anemia is intra- or intervascular.

Bone marrow evaluation for animal number 183623, terminated on day 154 of the study, is requested.

The data referred to in the letter of May 8th, in support of the observation that the increase in the erythroid series for the 4 mg/kg level is in the range of control dogs should be submitted. The controls of the five 1-year dog studies referred to in the May 8th letter should be included in the control population cited.

I. Mutagenic Studies

Review by John Chen for R. Landolt

A. Test Substance: Ethalfluralin. Chromosome Aberrations in Chinese Hamster Ovary Cells (CHO) In Vitro. Report No. LSR-RTC 095003-M-02385. August 23, 1985 (Authors: P. Mosesso, Brian Dean, and R. Forster). Accession No. 259342

Procedure:

1. Chemical Tested

The chemical name of Ethalfluralin (95.5% Purity) was given as N-ethyl-N-(2-methyl-2-propenly)-2,6 dinitro-4 (trifluoromethyl)benzenamine and N-ethyl- , , -Trifluoro-N-(2-methally)-2,6-dinitro-p-toluidine. Solutions of the test compound were prepared immediately before use in DMSO.

2. Cell Line

The Chinese Hamster Ovary cell line was obtained from Dr. F. Palitti, University of Rome, Italy. The CHO cell cultures were grown in Ham's F-10 medium supplemented with antibiotic solution, L-Glutamin (200 mm), sodium bicarbonate (7.5%) and 15 percent newborn calf serum at 37 °C in a 5 percent carbon dioxide atmosphere (100% humidity). Approximately 24 to 30 hours before treatment, an appropriate number of flasks (25 Sq. CM) seeded with 5 x 10^5 cells in supplemented Ham's F-10 were prepared for this study.

3. Metabolic Activation System

The in vitro metabolic activation system contained rat liver enzymes and an energy-producing system (cofactor solution) necessary for its function. The preparation of liver microsomes (S-9 fraction) from male Sprague-Dawley rats treated previously with phenobarbital and beta-naphthoflavone mixture was based on the method described by Ames et al. (Mutation Res. 31: 347-364, 1975). The final made up S-9 mix (10 mL) contained

3 mL of S-9 fraction, 1 mL of 40 mM NADP, 1 mL of 50 mM G-6-P, 2 mL of 200 mM Hepes, 1 mL of 40 mM $MgCl_2$, 1 mL of 330 mM KCl and 1 mL of distilled water.

4. Preliminary Toxicity Test

In order to establish the top dose to be used in the main cytogenetic assay, a preliminary toxicity test was performed. Ten concentrations were assayed: 0.12, 0.25, 0.50, 1.20, 2.50, 5.0, 12.0, 25.0, 50.0, and 120 ug/mL. The same assay methods were used as for the main assay.

5. Cytogenetic Assay Methods (Main Assay)

The CHO cells in the log phase of growth (5×10^6 cells/flask) were exposed to 3 concentrations of Ethalfluralin (7.24, 22.9, and 72.4 ug/mL) without metabolic activation for 24 hours at 37 °C. For the assay with metabolic activation, the CHO cells were exposed to 4 concentrations of the test compound (5.0, 15.8, 50.0, 85.0 ug/mL) for 3 hours at 37 °C. At the end of the exposure period, the mitotic cells were harvested by the metaphase shake-off method, swelled by hypotonic solution, and fixed in freshly prepared methanol: acetic acid fixative. Chromosome slides were prepared by dropping treated cells on clean, wet glass slides to produce metaphase chromosme spreads. The slides were stained in 3 percent Giemsa in Sorensens buffer and rinsed twice with distilled water.

In the absence of S-9 metabolic activation, the cells were treated for more than one cell cycle and were harvested after 24 hours. In the presence of S-9 metabolic activation, the treatment time was 3 hours, but two harvesting times (12 hours and 24 hours) were used. These cell cultures were prepared at each test point and 100 metaphases were scored from each culture. The untreated control, solvent control (DMSO) and the positive control compounds (mitomycin c and cyclophosphamide) were run concurrently in this study. The experiments were performed to comply with guidelines required by EEC Directive 79/831 Annex V Part B, OECD Guideline 473 and the principles of good laboratory practices for nonclinical laboratory studies as set forth by the U.S. FDA.

6. Statistical Analysis of the Data

The Fisher's Exact Test was used to compare the number of aberrations (assumed to have a Poisson distribution between cells) in the controls with the treated cultures.

Results:

1. Preliminary Toxicity Test

1. Preliminary Toxicity Test - continued

Treatment	Dose ug/ml	W/O S-9 Mix			W/S-9 Mix		
		Cell Scored	Meta-phases	Mean Mitotic Index(%)	Cell Scored	Meta-phases	Mean Mitotic Index(%)
DMSO	1%	1071	140	12.8	1161	97	7.7
		1043	130		1041	72	
Ethylfluralin							
	0.12	1018	107	11.3	1097	108	9.3
		1007	122		1009	89	
	0.25	1018	109	11.2	1000	69	8.6
		1001	117		1216	125	
	0.50	1066	123	11.7	1006	80	8.3
		1001	119		1000	87	
	1.20	1044	137	12.8	1016	94	8.5
		1004	125		1054	82	
	2.50	1004	96	11.1	1033	95	9.6
		1035	131		1000	101	
	5.00	1000	92	9.6	1005	73	7.9
		1015	102		1004	86	
	12.00	1100	145	11.9	1000	87	8.5
		1025	108		1071	89	
	25.0	1068	120	12.8	1039	79	7.3
		1019	147		1061	74	
	50.0	1069	111	8.8	1073	156	14.4
		1067	77		1015	145	
	120.0	1003	25	2.6	No cells recovered		
		1001	27				
Cyclo-phosphamide	26.3	1035	65	10.0	1012	16	2.0
		1006	138		1000	24	

Findings:

a) In the absence of S-9 metabolic activation, the test compound induced a dose-related reduction in mitotic index at the two highest dose levels (120 and 50 ug/mL). On this basis, the maximum dose level for the main assay in the absence of S-9 metabolism was selected as 72.4 ug/mL as a dose level calculated to produce a 50 percent in the mitotic index compared with the solvent control value.

b) In the presence of S-9 metabolic activation, the test compound demonstrated gross toxicity at the top dose level (120 ug/mL) and no cells were recovered after treatment. Therefore the maximum dose level for the main assay in the presence of S-9 metabolism was selected as 50 ug/mL (sub-toxic dose level). However, an additional higher dose level (85 ug/mL) in the main assay to ensure adequate testing was included in this study.

Results:

2. Summary of Chromosomal Aberrations in CHO Cells (Gaps not included)

Treatment without S-9 Metabolic Activation		No. of Cells Scored	Total No. of Aberrations Observed	No. of Cells Bearing Aberrations
Sampling Time: 24 hr				
Untreated		100	1 (B)	1
		100	1 (E,F)	1
	Total	200	2 (B,E,F)	2
Solvent (1% DMSO)		100	2 (E,F)	2
		100	0	0
	Total	200	2 (E,F)	2
Ethalfluralin				
7.24 ug/mL		100	1 (A,E)	1
		100	1 (B,E)	1
	Total	200	2 (A,B,E)	2
22.9 "		100	2 (B,E)	2
		100	2 (F)	2
	Total	200	4 (B,E,F)	4
72.4 "		100	0	0
		100	0	0
	Total	200	0	0
Positive Control		100	78 (A,B,C,D,E,F)	48***
(Mitomycin C-0.1 ug/mL)		100	80 (A,B,C,D,E,F)	45***
	Total	200	158 (A,B,C,D,E,F)	93
Treatment with S-9 Metabolic Activation				
Sampling Time: 12 hrs				
Untreated		100	1 (E,F)	1
		100	1 (C,E)	1
	Total	200	2 (C,E,F)	2
Solvent (1% DMSO)		100	1 (C)	1
		100	2 (E,F)	2
	Total	200	3 (C,E,F)	3
Ethalfluralin				
5.0 ug/mL		100	2 (B,F)	2
		100	0	0
	Total	200	2 (B,F)	2
15.8 "		100	0	0
		100	0	0
	Total	200	0	0
50.0 "		100	1 (C)	1
		100	0	0
	Total	200	1 (C)	1
85.0 "	No Metaphases			
Positive Control				
(Cyclophosphamide 6.6 ug/mL)		100	2 (E,F)	2
		100	2 (F)	2
	Total	200	4 (E,F)	4

Results:

2. Summary of Chromosomal Aberrations in CHO Cells - Cont'd

Treatment with S-9 Metabolic Activation		No. of Cells Scored	Total No. of Aberrations Observed	No. of Cells Bearing Aberrations
Sampling Time: 24 hr				
Untreated		100	1 (E,F)	1
		100	0	0
	Total	200	1 (E,F)	1
Solvent (1% DMSO)		100	4 (D,E,F)	3
		100	1 (E,F)	1
	Total	200	5 (D,E,F)	4
Ethalfluralin				
5.0 ug/mL		100	1 (A,E)	1
		100	1 (E,F)	1
	Total	200	2 (A,E,F)	2
15.8 "		100	1 (B,E)	1
		100	2 (E,F)	1
	Total	200	3 (B,E,F)	2
50.0 "		100	10 (B,D,E,F)	4
		100	4 (D,E,F)	3
	Total	200	14 (B,D,E,F)	7
35.0 "		100	73 (A,B,D,E,F)	31***
		100	57 (A,B,C,D,E,F)	29***
	Total	200	130 (A,B,C,D,E,F)	60
Positive Control (Cyclophosphamide 6.6 ug/mL)		100	19 (A,B,D,E,F)	15***
		100	9 (B,D,E,F)	8**
	Total	200	28 (A,B,D,E,F)	23

** Significantly greater than the solvent control $P < 0.05$;
*** Significantly greater than the solvent control $P < 0.001$;
(A) = Chromosome Deletion; (B) = Chromatid Deletion; (C) = Chromosome Exchange; (D) = Chromatid Exchange; (E) = Heavily Damaged Cells (more than 5 aberrations); (F) = Isochromatid or Isclocus Break.

Findings:

a) The positive control compound (Mitomycin C and Cyclophosphamide) induced significantly elevated levels of chromosome damages in all the experiments ($P < 0.001$ or $P < 0.05$) except the results obtained from the cyclophosphamide-treated sample after 12 hours.

b) In the absence of S-9 metabolism the test compound did not induce any statistically significant increases in the number of cells bearing aberrations. However, in the presence of S-9 metabolism the test compound induced a highly significant increase ($P < 0.001$) in the number of cells bearing aberrations at the top dose level (85 ug/mL, 24 hours sampling time). Therefore, the test compound, Ethalfluralin, produced sufficient evidence to be considered clastogenic in the presence of S-9 metabolism when assayed in CHO cells under the reported experimental conditions.

Evaluation:

The assay appears to have been conducted in a manner to generate valid results. Ethalfluralin is considered clastogenic in the in vitro cytogenetic assay in Chinese hamster ovary cells with the S-9 metabolic activation. However, a minor deficiency for determining the maximum dose level (50 ug/mL) for the main assay in the presence of S-9 metabolism is noted. The study is considered acceptable.

B. Test Substance: Ethalfluralin. Forward Mutation in Schizosaccharomyces pombe P-1 LSR-RTC Report No. 095005-M 03085. August 23, 1985 (Authors: E.R. Adams, C.N. Edwards, and R. Forster). Accession No. 259342

Procedure:

1. Tester Strain

The haploid yeast strain Schizosaccharomyces pombe P-1 which carries a mutation in a gene involved in the adenine biosynthesis pathway (ade 6-60) and also the rad 10-198 mutation (deficient in DNA repair pathway) was used to measure the induction of forward mutation induced by Ethalfluralin with and without metabolic activation. A mutation occurring in any of the preceding five genes (ade 1, ade 3, ade 4, ade 5, and ade 9) prevents the accumulation of the red pigment, and the double mutant thus formed produces white colonies, which can readily be scored.

2. Metabolic Activation System

The in vitro metabolic activation system contained rat liver enzymes and an energy-producing system (cofactor

solution) necessary for its function. The preparation of liver microsomes (S-9 fraction) from five Sprague-Dawley rats induced previously with Aroclor 1254 was based on the method described by Ames et al. (Mutation Res. 31: 347-364, 1975). The final made up S-9 mix (10 mL) contained 2.5 mL of S-9 tissue fraction, 30 mg of NADP, 30 mg of G-6-P, 0.1 mL of $MgCl_2$ (0.4M) and KCl (1.65M) and 7.4 mL of phosphate buffer (0.1M).

3. Preliminary Toxicity Test

Since the test compound was found to be soluble in the incubation mixture at a concentration of 200 ug/mL, the top dose of 200 ug/mL was used together with four lower doses (63.2, 20.0, 6.32, and 2 ug/mL) in this toxicity test. The toxicity value was expressed as the percentage of control growth.

4. Forward Mutation Assay Method

Liquid culture of S. pombe P-1 was grown in YEA medium for 24 hours at 32 °C and should reach 5 to 10 million cells/mL. The yeast cells were harvested and resuspended for each test point as follows:

a) Yeast cell suspension: 0.9 mL;

b) One mL of phosphate buffer (0.01 M, pH 7.4), or where S-9 metabolic activation is required, 1 mL of S-9 mix;

c) Each of the test concentrations (12.5, 25, 50, 100, 200, and 300 ug/mL) or control substances (MMS and CP was added as 0.1 mL) or where organic solvent (DMSO) is used at 0.05 mL together with 0.05 mL of phosphate buffer.

These incubation mixtures prepared in universal bottles were incubated for 16 hours at 32 °C in a shaking water bath. At the end of incubation period, the contents of each bottle were diluted with 20 mL of sterile distilled water, harvested by centrifugation, and again resuspended in 2 mL of YEL medium which were used for plating purpose.

Ten YEA plates were inoculated with approximately 3000 cells/plate to estimate mutant numbers. Three YEA plates were inoculated with a fixed dilution of the yeast suspension to estimate survival levels. Three other plates were inoculated with approximately 300 cells/plate to estimate the plate efficiency of the cells. The plates were incubated for 5 days at 32 °C. After the incubation period, the plates may be held at 4 °C prior to scoring. Scoring was effected by counting the number of colonies on the plating efficiency plates and the number of white or sectored colonies on the mutation plates.

5. Statistical Analysis

The statistical significance of the data was assayed in two ways:

a) At each dose level, the number of mutant colonies obtained was compared with the control value using the modified Chi-Squared statistic.

b) After square root transformation of the data to satisfy normal distribution and homoscedasticity assumptions, a linear regression equation was fitted to the data using the least squares method. A statistical significant regression line with a positive slope will be taken as evidence of a dose-response relationship.

Results:

1. Preliminary Toxicity Test (16 hours of growth on YEA medium)

Treatment	Dose	Without S-9 Mix		With S-9 Mix	
		Plate Count*	Percentage Survival**	Plate Count*	Percentage Survival*
Untreated (Distilled water)	-	226	90	268	82
Solvent Control (DMSO)	2.5%	252	100	326	100
Ethalfluralin	2 ug/mL	278	111	254	78
	6.32 "	264	105	280	86
	20 "	174	69	306	94
	63.2 "	206	82	270	83
	200 "	181	72	214	66

* Mean value obtained from three plate counts;
** Percentage Survival: Mean plate counts expressed relative to solvent control.

Findings: The test compound was found to be slightly toxic to the treated cells at the highest dose level (200 ug/mL) in the presence and absence of S-9 metabolism. On the basis of these results, a maximum dose level of 200 ug/mL was selected for the main assay. However, an additional higher dose level (300 ug/mL) in the main assay to ensure adequate testing was included in this study.

Results:

2. Summary of Forward Mutation in Schizosaccharomyces Pombe P1

Treatment	Without S-9 Mix - Colony Counts				With S-9 Mix - Colony Counts			
	Survival Plate*	Efficiency Plate*	Mutants Comp.	Mutants Sect.	Survival Plate*	Efficiency Plate*	Mutants Comp.	Mutants Sect.
Exp. 1								
Untreated	252	211	2	2	239	215	1	0
Distilled water	211	211	2	2	215	215	1	0
Positive Controls								
MMS, 0.07 ug/mL	285	283	7***	33***				
0.14 "	188	229	6***	58***				
CP, 1.25 "					167	213	2	7**
2.50 "					132	224	1	19***
Ethalfluralin								
0 ug/mL	211	211	1	0	239	239	0	2
12.5 "	259	305	1	1	283	298	0	2
25.0 "	205	205	3	1	276	260	1	0
50.0 "	260	286	0	0	230	284	0	1
100 "	206	217	2	1	167	282	0	1
200 "	193	178	1	1	224	263	0	1
300 "	147	302	1	0	207	266	0	2
Exp. 2								
Untreated	284	325	1	0	286	265	0	0
Distilled Water	325	325	1	0	265	265	0	0
Positive Controls								
MMS, 0.07 ug/mL	195	242	9***	35***				
0.14 "	164	207	14***	34***				
CP, 1.25 "					170	249	0	15***
2.50 "					132	196	1	21***
Ethalfluralin								
0 ug/mL	228	228	0	1	245	245	0	1
12.5 "	218	259	0	1	278	253	0	2
25.0 "	304	304	0	0	255	267	2	0
50.0 "	224	232	0	0	175	254	1	0
100 "	174	246	1	1	185	212	0	0
200 "	102	151	0	2	124	210	0	0
300 "	93	111	1	1	170	337	0	0

* Mean value obtained from three plate counts;
** Significantly greater than the solvent control $P < 0.05$;
*** Significantly greater than the solvent control $P < 0.001$;
MMS = Methylmethansulphonate (Statistical Regression Line: $Y_1 = 1.5178 + 27.9341X$; $Y_2 = 1.0820 + 30.4338\ X$);
CP = Cyclophosphamide (Statistical Regression Line: $Y_1 = 0.7556 + 0.9226\ X$; $Y_2 = 0.2602 + 1.3390\ X$);
Comp. = Complete mutants scored from 10 plates;
Sect. = Sector mutants scored for 10 plates.

2. Summary of Forward Mutation in S. pombe P1 - continued

Findings:

1) The positive control compound (MMS and CP) induced expected frequencies of forward mutation in S. pombe P1 that were greatly in excess of the vehicle control values the nonactivation and activation systems ($P < 0.05$ or $P < 0.001$). These positive responses indicated that the assay systems were functioning properly.

2) The test compound did not induce forward mutation in S. pombe P1 either in the presence or absence of S-9 metabolism under the reported experimental conditions.

Evaluation:

The assay appears to follow the general procedures of S. pombe (P1) mutagenicity test recommended by Loprieno N. et al. (Testing of chemicals for mutagenic activity with Schizosaccharomyces pombe: A Report of the U.S. EPA Gene-Tox Program. Mutation Res. 115:215-223, 1983). However, the following deficiencies in reporting of this study should be clarified:

1) Details of procedures used for the preparation of liquid culture of S. pombe P1 prior to testing by the selection of red-purple colonies were not presented in this report.

2) Appropriate culture media for culture growth and for the determination of survival and mutant colonies were not clearly described.

The study is unacceptable in the present form. However, this study may be upgraded to acceptable on resolution of the reporting deficiencies.

C. A Summary of Genetic Toxicology Studies with Ethalfluralin Lilly Research Laboratories, August 1985 (Prepared by G.S. Probst) Accession No. 259342

Registrant's Conclusion:

Ethalfluralin was previously evaluated for potential genotoxicity in a battery of in vitro and in vivo tests (Appendix A) which were conducted at Lilly Research Laboratories at Greenfield, Indiana. Since Ethalfluralin has been evaluated in four mammalian assays (Hepatocyte DNA Repair; L5178Y Point

Mutation; In Vivo SCE; Rat Dominant Lethal) for mutagenicity/ genotoxicity without any evidence of a positive effect, the positive findings of in vitro chromosome aberrations in CHO cells reported by LSR are less meaningful. Therefore, "it is reasonable to conclude that Ethalfluralin does not pose a mutagenic hazard to mammalian systems."

Toxicology Branch Recommendation:

Toxicology Branch acknowledges receipt of the following mutagenicity studies with Ethalfluralin previously submitted by the Registrant:

Mutagenic - Ames; Lilly Res. Labs.; #LBMS 1169	Non-mutagenic at concentration ranging from 1000-0.1 ug/mL +/- activation in Salmonella strains: G46, TA1535, TA100, C3076, TA1537, D3052, TA1538, TA98, and E. coli strains: WP2 and WP2 uvr A	Unacceptable 002251
Mutagenic - Salmonella; Lilly; #83404GPA1169; 6/83	Positive for increased revertants in Salmonella and E. coli. (TA1535, TA100, TA98) Tested with and without metabolic activation. Doses tested: 0.1 - 1000 ug/mL	Acceptable 003269
Mutagenic - Salmonella; Lilly; #830307AMS1169, 830404AMS1169 & 830425 AMS1169; 5/83	Positive for increased revertants in Salmonella. Strain tested: TA1537, TA1538, TA1535, TA98 and TA100; positive in TA1535 - activated, TA100 (dose response) +/- activation. Dose tested: 125-1000 ug/mL	Acceptable 003269
Mutagenic - DNA repair in rat hepatocytes Lilly; #791120-263; 6/80	Negative for UDS (repair) up to toxic doses (500, 1000 nM) Doses tested: 0.5, 1.0, 5, 10, 50, 100, 500, 1000 nM/mL	Acceptable 003269
Mutagenic - gene mutation in mouse lymphoma cells; Lilly; #830208MLA1169; 4/83	Negative for TK locus in L5178Y cells up to toxic doses. Doses tested: 0.1, 0.25, 0.5, 0.75, 1.0, 2.5, 5.0, and 10 ug/mL	Acceptable 003269

Mutagenic - DNA repair in bone marrow; Lilly; #830214SCE1169; 3/83	Negative for SCE induction in females (males not tested) Doses tested: 200, 300, 400, 500 mg/kg. Cytotoxic at 400 and 500 mg/kg.	Unacceptable 003269
Mutagenic - dominant lethal - rat; Lilly Res. Lab.; #R-159; 12/80	No evidence of dominant lethal efffect. Dose tested: 5.0 g/kg (25%) suspension in 10% aqueous acacia solution	Unacceptable 002251

Based on the mutagenicity studies with Ethalfluralin submitted previously by Registrant, the in vivo SCE assay and the dominant lethal test in rats were judged inadequate to be acceptable. Until the reporting deficiencies cited in our conclusions in the previous reviews for the dominant lethal test in rats (TB memorandum October 29, 1982 Roland Gessert and Irving Mauer) and the in vivo SCE assay in rats (TB memorandum September 28, 1983 Irving Mauer) are clarified and resolved, we are unable to determine the potential genotoxicity of the test compound in mamalian systems.

II. Teratology - Rat

Bio-Research Lab No. 82182, November 1985, Acc. No. 260434

A. Ethalfluralin* (95.5%) was administered orally in a 10 percent aqueous acacia suspension at 10 mL/kg to four groups of 25 bred Charles River female rats at 0, 50, 250, and 1000 mg/kg daily on days 6 through 15 of gestation. The female rats were 73 days of age and weighed between 209 and 240 g. Evidence of mating was determined by the presence of spermatozoa in the vaginal lavage. Body weights were recorded on days 0, 6, 11, 16, and 20 of gestation. Food consumption was measured daily on days 0 to 20 of gestation. All animals were examined daily with urine color verified on days 6 and 7, 11 and 12, and 15 and 16 of gestation. The study was terminated on day 20 of gestation and the females were subjected to a gross pathological examination. At the time of cesarean section, fetal examination consisted of number and position in utero of live and dead, number and position of resorption sites, number of corpora lutea,

*Identified as EL-161, Compound No. 94961, Batch No. B30-Y64-35B was reported to contain 0.09 ppm of N-ethyl-2-methyl-N-nitroso-2-propen-1-amine.

sex, body weight, and gross observations. Fetal heads were placed in Bouins fluid and examined by the technique of Wilson. Half of each litter was examined for soft tissue alterations. All fetuses were examined for skeletal alterations. Reporting of fetal findings were categorized as major malformation, minor anomalies and common variants. For each litter, calculations were made for preimplantation loss (%), postimplantation loss (%), resorptions (%), and live fetuses (%).

B. Results

1. Analytical Chemistry

a. The mean assayed concentration (mg/mL) of ethalfluralin in suspension was 4.32-6.23(5), 22.2-23.02(25) and 93.08-94.7(100) with the theoretical concentration indicated in parenthesis.

2. Maternal Observations

a. Mortality - No deaths reported for any dose level.

b. Clinical Findings -

i. The urine of females treated at the 250 and 1000 mg/kg dosage levels appeared dark on day 7 of gestation and decreased in severity by day 16 of gestation. Yellow staining of the urogenital area was also observed at the 1000 mg/kg level.

c. Body Weight

i. At the high level (1000 mg/kg) a 45 percent decrease in the group mean body weight gain was observed during days 6 through 11 of gestation followed by a gradual increase in the mean body weight gain during days 11 through 16 of gestation and was comparable to the control values by the termination of the study.

ii. The absolute mean body weight gain of the 50 and 250 mg/kg levels was comparable to the control values.

iii. "The corrected body weights and the corrected body weight gains in the 250 and 1000 mg/kg/day groups were significantly decreased ($P < 0.05$ and $P < 0.001$, respectively)." The percent differences in the corrected body weight gain during days 0 through 20 of gestation, between the control and the 50, 250 and 1000 mg/kg/day test levels were 6, 11, and 15 percent, respectively.

d. Food Consumption

i. At the high level (1000 mg/kg) a 14 percent decrease in the group mean food consumption was observed during days 6 through 11 of gestation followed by a 6 percent decrease during days 11 through 16 of gestation and was comparable to the control values by the termination of the study.

ii. The group mean food consumption of the 50 and 250 mg/kg levels were comparable to the control values.

e. Gross Pathological Findings

i. No gross pathological findings related to the administration of the test material were observed.

f. Pregnancy rate was 100 percent in all groups except for the 250 mg/kg level which was 92 percent.

3. Uterine Observations

a. The following parameters recorded for all three test levels were comparable to the control observations: number of corpora lutea, implantation sites, resorptions, pre- and postimplantation losses and the percentage of resorptions.

b. Fetal Observations

i. No dead fetuses were recorded.

ii. The group and litter mean body weights and sex ratios of the test levels appear unaffected as compared to the control values.

iii. Major Malformations

The high level (1000 mg/kg) was without incidence of major malformations with one fetuses in each of the two lower dose levels and control observed to have a major malformation.

iv. Minor Anomalies

A significant increase ($P < 0.05$) in the incidence of litters with minor skeletal anomalies was reported for the 1000 mg/kg/day level. This represents 53 fetuses affected in 23 litters as compared to the control values of 42 fetuses affected in 16 litters. With reference to the attached table for comparison the group incidence of minor skeletal anomalies reported in this study are within the range of values observed in the seven historical data studies conducted between

1983 and 1984. The group litter mean percentage of affected fetuses were not significantly different from the control values. This increase in minor skeletal anomalies does not appear to be related to the administration of the test material.

v. Common Variants

The incidence of common skeletal variants (thoracic and sternebral) were similar in occurrance between the test and control groups.

C. Conclusion

1. Classification of Data - Guideline

2. The incidence of hydronephrosis reported previously (Gessert October 29, 1982) in the rat teratology study (250 mg/kg HLT) conducted by Lilly Research Labs, No. R0 6880, 1980 (Acc. No. 070682) was not evident in this study.

3. Maternal NOEL 50 mg/kg/day

Maternal LOEL 250 mg/kg/day with a significant decrease in the corrected body weight gain and the elimination of dark urine observed during days 7 through 16 of gestation.

Developmental NOEL 1000 mg/kg/day

Group Incidence of Minor Skeleted Anomalies

	GROUP 1 VEHICLE CONTROL 10 ML/KG/DAY		GROUP 2 ETHALFLURALIN 50 MG/KG/DAY		GROUP 3 ETHALFLURALIN 250 MG/KG/DAY		GROUP 4 ETHALFLURALIN 1000 MG/KG/DAY	
	NO. OF LITTERS EXAMINED	NO. OF FETUSES EXAMINED	NO. OF LITTERS EXAMINED	NO. OF FETUSES EXAMINED	NO. OF LITTERS EXAMINED	NO. OF FETUSES EXAMINED	NO. OF LITTERS EXAMINED	NO. OF FETUSES EXAMINED
SKELETAL/EXTERNAL	25	349	25	355	23	337	25	374
INTERNAL	23	171	25	177	23	166	25	185
TECHNIQUE OF WILSON	23	171	25	178	23	166	25	185
	NO. OF LITTERS AFFECTED	NO. OF FETUSES AFFECTED	NO. OF LITTERS AFFECTED	NO. OF FETUSES AFFECTED	NO. OF LITTERS AFFECTED	NO. OF FETUSES AFFECTED	NO. OF LITTERS AFFECTED	NO. OF FETUSES AFFECTED
MINOR SKELETAL ANOMALIES (TOTAL)+	16	42	18	51	19	65	23*	53

*P<0.05

Historical Control Data (1983-1984)
Group Incidence of Minor Skeletal Anomalies

	NO. OF L/E	NO. OF F/E	NO. OF L/E	NO. OF F/E	NO. OF L/E	NO. OF F/E	NO. OF L/E	NO. OF F/E	NO. OF L/E	NO. OF F/E	NO. OF L/E	NO. OF F/E	NO. OF L/E	NO. OF F/E	NO. OF L/E	NO. OF F/E
TERNAL	26	368	23	327	23	295	21	282	23	328	22	300	22	274	20	254
TERNAL	26	253	23	229	23	253	21	282	23	110	22	149	22	139	20	124
LETAL	26	253	23	229	23	253	21	282	22	315	22	300	22	272	20	251
NIQUE OF WILSON (HEADS)	26	121	23	110	23	86	21	190	23	109	21	149	22	134	20	124
	NO. OF L/A	NO. OF F/A	NO. OF L/A	NO. OF F/A	NO. OF L/A	NO. OF F/A	NO. OF L/A	NO. OF F/A	NO. OF L/A	NO. OF F/A	NO. OF L/A	NO. OF F/A	NO. OF L/A	NO. OF F/A	NO. OF L/A	NO. OF F/A
OR SKELTAL ANOMALIES (TOTAL)+	18	50	18	48	14	43	17	84	20	52	10	23	16	48	18	52

III. Chronic Oral Toxicity-Dog

Lilly Research Laboratories No. DO 1684, November 1985, Acc. No. 260434

A. Procedure

Thirty-two 7- to 8-month-old male and female beagle dogs weighing 8.3 ± 0.18 kg and 7.3 ± 0.19 kg, respectively, were divided into four groups of four animal per sex per dosage level of 0, 4, 20, and 80 mg/kg. The test material (95.5%) identified as compound 94961 or EL-161, batch number 830-Y64-35B* was administered orally in capsules daily for one year. Body weights were recorded initially and at weekly intervals with food consumption estimated each day. All of the animals received an ophthalmic examination initially, then at 6 and 12 months. Hematologic determination were made initially, at 1 and 3 months, then monthly thereafter for the duration of the study to include: hemoglobin, PCV, MCV, MCH, MCHC, activated partial thromboplastin time, thromocyte count, erythrocyte count and morphology, and total and differential leukocyte counts. A cytologic evaluation of bone marrow smears including an estimated M:E ratio was performed at the termination of the study. Clinical chemistry determinations were made initially, then at 1, 3, 6, and 12 months to include: glucose, urea nitrogen, creatinine, total bilirubin, alkaline phosphatase, alanine transaminase, and cholesterol. Urinalysis determinations were made initially, then at 1, 3, 6, and 12 months to include: specific gravity, pH, color, content of protein, glucose, occult blood, ketones, bilirubin, and urobilinogen. Organ weights were recorded for brain, liver, kidney, heart, thyroid, adrenals, testes, and ovaries. Representative portions of all tissues and organs of all animals were collected for histopathologic examination.

B. Results

1. Gross Observations

a. Signs of Toxicity

One incidence of mucoid stools was reported for one control dog during the 12th month of the study. Soft mucoid stools for 2/8 and emesis for 1/8 animals were observed

*Reported to contain the impurity N-ethyl-2-methyl-N-nitroso-2-propen-1-amine in the concentration of 0.09 ppm.

at the 4 mg/kg dose level once during the third and fifth month of the study. At the 20 mg/kg level emesis was observed for three dogs and soft stools for four dogs once during the 6th, 10th, and 11th month. One animal of the 80 mg/kg level remained normal in appearance throughout the study. The other dogs of the high level exhibited soft and/or mucoid stools for 2 to 7 days during the study. One female (No. 183263) of the high level was euthanized on day 153 of the study. This dog was hypoactive, ataxic with tremors during the third month, and removed from treatment for 6 days after which treatment was resumed. During the fifth month this animal was observed unconscious without pupillary light response, abdominal muscles tense, limb muscles rhythmically contracting, and emesis occurred prior to euthanization. One other dog, a male (No. 184193) of the 80 mg/kg level was observed during the fourth month to have soft mucoid stools containing a reddish-brown fluid, pale mucous membrane, abduction of the hind limbs, rapid, weak pulse, in poor condition, and not eating. Neurological examination of this animal was negative. After 2 days without treatment this animal (No. 184193) returned to normal and remained in the study in good health.

b. Food Consumption

Three dogs (Nos. 183623, 183633, and 184193) dosed at 80 mg/kg were off food from 1 to 6 days during the study. The male described previously (No. 184193) for gross observations was off food on several occasions from 1 to 3 days, for a total of 11 days, during the study but maintained a comparable body weight to the other males dosed at the high level. The other animal, a female (No. 183633), at the high level, was off food for 2 days during the study but maintained its body weight comparable to its pretreatment weight for the duration of the study.

c. Body Weight

A significant decrease in the mean body weight gain was reported for the males of the 80 mg/kg level by the second week and remained depressed for the duration of the study. The group mean body weight gain of the females of all three dosage levels was comparable to the controls.

d. Opthalmic examination was negative for all three test and control dogs examined at the 6-month interval and at the termination of the study.

2. Clinical Evaluation

a. Clinical Blood Chemistry

A significant decrease in blood glucose levels was reported for males of the 80 mg/kg dosage level by day 85 and for males of the 20 mg/kg dosage level by day 176, both values were comparable to the control values by the termination of the study. A transient, but significant increase in the total bilirubin values for males and females of the 20 and 80 mg/kg dosage levels was reported by day 29 becoming comparable to the controls by day 176. Alkaline phosphatase values increased to greater than 50 percent by day 29 for the females of the 20 and 80 mg/kg level and for males of the 80 mg/kg level by day 176, remaining elevated for the duration of the study. A significant decrease in male and female creatinine values was observed for the 20 and 80 mg/kg level by day 29 being comparable to control values for the duration of the study.

b. Urinalysis

The urine appeared amber* and clear on day 30 for males of the 20 mg/kg level and for females of the 80 mg/kg level. The urine of the female dog (No. 183623) euthanized on day 153 of the study was described as black cloudy with a 3+ for protein, trace glucose, 3+ occult blood, and 1+ for ketones and bilirubin.

An increase in the incidence of bilirubin in the urine collected from dogs of the 20 and 80 mg/kg level was observed as compared to the controls.

c. Hematology

By day 85 of the study, the thrombocyte counts for males and females of the 80 mg/kg level were significantly elevated accompanied by an observed increase of 20 to 40 percent for males and females of the 20 mg/kg level,† remaining elevated for the duration of the study. The

*Amber urine was reported to be indicative of ethalfluralin metabolism.

†These increases in the thrombocyte count observed at the 3-month interval for animals in the 20 and 80 mg/kg levels prompted an alteration in the sampling of the hematology parameters from 6 and 12 months to monthly intervals for the duration of the study.

thromboplastin time of all three test levels was comparable to the control values.

At the 80 mg/kg level, male mean erythrocyte, hemoglobin, and packed-cell volume values were significantly depressed by day 118 through to termination of the study. By day 85, female erythrocyte, hemoglobin, and packed-cell volume values of the 20 and 80 mg/kg levels were significantly depressed, being comparable to the control values by day 118 through to end of the study. A significant decrease in male and female mean corpuscular hemoglobin concentration (MCHC) was observed at the 80 mg/kg level during the 273- to 301-day intervals for females from the 29- to 147-day interval and at the termination of the study. An increase in the ratio of nucleated erythrocytes to 100 leukocytes was reported 1/4 females of the 20 mg/kg level, 1/4 males and 1/4 females (terminated day 153) of the 80 mg/kg level. Variations in the elevation of the leukocyte count of males and females of the 80 mg/kg level were observed with increases of 20 to 77 percent during days 85 through 176 and on day 301, being comparable to the control values during the other intervals.

At the 80 mg/kg level, male lymphocyte count was significantly depressed on days 147 to 210 and the female lymphocyte count was depressed significantly on days 85 to 232. A transient but significant decrease in female lymphocyte count was reported on day 147 for the 20 and 4 mg/kg levels followed by a return to normal values by day 210.

A significant increase in the male neutrophile count was reported for the 80 mg/kg level by day 85 through day 322 being comparable to the controls values by the termination of the study. Female neutrophile count was significantly elevated at the 80 mg/kg level by day 85 being comparable to the control values for the duration of the study.

A significant decrease in band neutrophil count was reported for males of all three dose levels on day 322. By day 210 a 40 to 60 percent decrease in eosinophil count was reported for males of all three dosage levels being significant at the 80 and 4 mg/kg levels.

Number of Animals Showing an Incidence of Erythrocyte Morphology in Study DO 1684

Variation Observed	Treatment Group: Control M	Control F	4 mg/kg M	4 mg/kg F	20 mg/kg M	20 mg/kg F	80 mg/kg M	80 mg/kg F
Normal	4/4	4/4						
Anisocytosis			2/4	2/4		2/4	2/4	2/4

Variation Observed	Control M	Control F	Treatment Group 4 mg/kg M	4 mg/kg F	20 mg/kg M	20 mg/kg F	80 mg/kg M	80 mg/kg F
Polychromasia			2/4	2/4	3/4	3/4	3/4	4/4
Poikilocytosis			1/4	2/4	2/4			
Macrocytes						3/4	3/4	4/4
Target cells							1/4	
Howell-Jolly Bodies					3/4	2/4	4/4	4/4
Hypochromia							1/4	
Rouleaux							1/4	

Male and female pretest erythrocyte morphology was normal for controls and all three dosage levels except for an incidence of polychromasia (1/4) and poikilocytosis (1/4) observed for females of the 4 mg/kg level.

Erythrocyte morphology was normal for the controls with variations in size and color reported for all three dosage levels. The presence of macrocytes was observed for females of the 20 mg/kg level and for males and females of the 80 mg/kg level. The number and frequency of occurrence of Howell-Jolly Bodies in the 20 and 80 mg/kg levels were greator than observed for the pretest or control animals from day 29 to 85 through to the termination of the study.

Prior to the completion of this review, additional data were received with Elanco Products Company letter of May 8th, 1986, presenting the incidence of erythrocyte morphology observed in the pretreatment, control and test levels of five 1-year dog studies conducted at Lilly Research Laboratories. The results of the pretreatment observations are presented in the following table for comparison to the study under review (DO 1684).

Pretreatment Incidence of
Erythrocyte Morphology Change

Study No.	Sex	Number of Observations	Anisocytosis	Poikilocytosis	Polychromasia
DO 2683	Male	72	1		
	Female	72			
DO 4883	Male	48		1	1
	Female	48			3
DO 6184	Male	48	1	1	1
	Female	48			

Study No.	Sex	Number of Observations	Anisocytosis	Poikilocytosis	Polychromasia
DO 1784	Male	48			1
	Female	48			2
DO 1785	Male	48			
	Female	48		2	
DO 1684	Male	48			
	Female	48		1	1

The changes in eryhthrocyte morphology observed during the pretreatment phrase of this study (DO 1684) are consistent with those values reported for these five studies.

The 4 mg/kg dosage level appears equivocal for alterations in erythrocyte morphology. In response to this concern the following observations were tabulated from the control population of the five 1-year dog studies conducted at Lilly Research Laboratories. These observations along with the control and 4 mg/kg level incidence of erythrocyte morphology are presented.

Control Incidence of
Erythrocyte Morphology Change

Study No.[1]	Sex	Number of Observations	Anis.	Poik.	Polyc.	Macrocytes	Rouleaux
DO 2683	Male	84	1(4)	1(5)	1(4)		
	Female	84	2(1,5)	1(5)	2(1,8)		
DO 4883	Male	46					
	Female	56		1(10)		1(6)	1(8)
DO 6184	Male	56					
	Female	56		4(4,4,10,10)	1(11)		

( ) = month variation was observed

[1]Data are listed for historical control animals, except for the last entry which shows the response in the present 4 mg/kg dose group.

Study No.[1]	Sex	Number of Observations	Anis.	Poik.	Polyc.	Macrocytes	Rouleaux
DO 1784	Male	56					
	Female	56	1(7)	1(5)	1(6)		
DO 1785	Male	56					
	Female	56	2(6,11)	2(11,12)	2(6,12)	2(6,11)	
DO 1684	Male	48					
	Female	48					
DO 1684 (4 mg/kg)	Male	48	2(2,10)	1(10)	3(3,10,10)		
	Female	48	2(10)	2(10)	2(10)		

( ) = month variation was observed

[1]Data are listed for historical control animals, except for the last entry which shows the response in the present 4 mg/kg dose group.

The incidence of erythrocyte morphology changes at the 4 mg/kg level appear significant when compared to the absence of variations in the controls of this study. However, when compared to incidence of occurrence in the population controls of these five studies and with consideration given to the lack of hematological changes at the low level of this study, these morphological changes do not appear significant. The occurrence of morphological changes in all of these studies is most apparent during the later 6 months of the respective studies.

3. Terminal Examination

a. The organ-to-body-weight changes were limited to the high dosage level with the two lower dosage levels comparable to the control values. Liver-to-body-weight and liver-to-brain-weight ratios of males and females of the 80 mg/kg level were significantly increased. Male kidney-to-body-weight and kidney-to-brain-weight ratios were increased by 36 and 16 percent, respectively for the 80 mg/kg level. Increases in male adrenal weights relative to body and brain weights of 30 to 50 percent were reported for the 4 and 80 mg/kg levels, but not for the 20 mg/kg level. A 27 to 44 percent (not dose related) increase in female adrenal weight relative to body weight was reported for all three dosage levels. Spleen weights were not recorded for this study.

b. Gross necropsy observations were limited to the female dog (No. 183623) that was euthanized after 153 days on test. Transudate in the pericardial sac, mottled liver, and brown contents of the urinary bladder were observed.

c. Histopathologic Findings

On day 153 of the study female number 183623 of the 80 mg/kg level was euthanized. Histopathological observations of this dog were lymphocytic necrosis of the thymus, lymph node, peyers patches, and gallbladder accompanied by lymphoid depletion of the spleen. Moderate thyroid hyperplasia (C-cell), slight centrilobular vacuolation, and siderosis of liver were reported for the same female. Evaluation of the sciatic nerve and spinal cord of this animal were negative.

At the termination of the study marked siderosis of the liver of one female of each of the 20 and 80 mg/kg levels with slight to minimal liver siderosis of two males and two females of the 80 mg/kg level was observed as compared to slight to minimal siderosis of one female control and one male of the 4 mg/kg level. Cortical tubular brown pigmentation (bilirubin deposition) of the kidney was observed for 5/7 dogs of the 80 mg/kg level as compared to the incidence of 3/8 in the controls, 4 mg/kg and 20 mg/kg levels. Mammary gland hypoplasia was not observed for the female controls or test levels. However, the incidence of male mammary gland hypoplasia of all three dosage levels was comparable to the control males. Thyroid hyperplasia (C-cell) was observed in 6/8 dogs of the 80 mg/kg level as compared to the control incidence of 4/8.

Histopathologic observations of the bone and bone marrow of all test and control animals including female dog number 183623 of the 80 mg/kg level were reported to be normal.

d. Bone Marrow Evaluation

	Treatment Group							
	Control		4 mg/kg		20 mg/kg		80 mg/kg	
Observation	M	F	M	F	M	F	M	F
Decreased seg. neutrophils	0/4	0/4	1/4	0/4	0/4	0/4	0/4	0/4
Increased erythroid series	0/4	0/4	0/4	1/4	1/4	1/4	3/4	3/4*

*Not reported for animal number 183623, terminated on day 154.

Control values for males and females were reported to be normal. An increase in the erythroid series was reported for 1/8 dogs of the 4 mg/kg level, 2/8 dogs of the 20 mg/kg level and 6/7 dogs of the 80 mg/kg level. Results of the bone marrow impression for female (No. 183623) dog of the 80 mg/kg level was omitted from table 5 of this report.

The supportive data that accompanied the Elanco letter of May 8, 1986 was limited to the incidence of hematological and erythrocyte morphology changes observed in five 1-year dog studies with the assurance that "the estimated M:E ratios for this dog group (4 mg/kg) were in the range of the control dogs of this laboratory and the bone marrow would be considered functionally normal." The shift in the M:E ratio of one animal of the low level may be more representative of the historical controls than for the controls of this study. This observation coupled with the comparable incidence of the erythrocyte morphology changes at the low level to the historical controls and the lack of hematological changes at the low level suggests that the 4 mg/kg level is the no-effect level for this study when compared to the historical control values rather than the controls of this study. However, this assumption should be supported by the data on the bone marrow evaluation of the control population to include the controls of the five 1-year dog studies cited in the May 8th letter.

C. Conclusions

1. Classification of Data - Supplemental.

a. Deficiency

(1) Spleen organ weights were not recorded.

(2) Bone marrow evaluation for animal number 183623, terminated on day 154 of the study was not reported.

(3) The data referred to in support of the observation that the increase in the erythroid series for the 4 mg/kg level is in the range of control dogs should be submitted. The controls of the five 1-year dog studies referred to in the May 8th letter should be included in the control population cited.